NATIONAL AERONAUTICS AND SPACE ADMINISTRATION

PROGRESS REPORT

Report Prepared by:	Walter H. Moran, Jr., M.D.
For Period:	February 15, 1966 to September 20, 1966
Grant:	NGR-49-001-019
Title:	The Effect of Changing Gravity and Weightlessness on Vasopressin Control Systems

A. ACQUIRING FACILITIES AND PERSONNEL:

Soon after the grant was awarded, the orders for laboratory benches and storage cabinets were placed. The LINC-8 digital process controller was also ordered and will arrive in November 1966. This laboratory computer will be used in setting up the additional rat antidiuretic hormone (ADH) bioassay systems required for this project and in the studies of hydration control that were mentioned in the previous research grant proposed.

In order not to delay the project while awaiting delivery of equipment, studies on the stability of ADH in blood were begun by borrowing the technical services of Mrs. K. Randall and Mr. T. Browne and the use of one of the rat ADH bioassay systems that was currently in operation.

We were extremely fortunate in obtaining Mrs. D. Brannon as our Research Technician on June 13, 1966. She holds a B.S. in Chemistry, has previously worked in this laboratory from December 1963 to July 1965, and is familiar with all of the technical procedures involved in the isolation of ADH. Dr. J. Hoppenstein, a second year surgical resident, joined the group in July 1966 as a Research Fellow. During the summer months Mr. R. Huber and Mr. J. Kessel, who were premedical students at the University, assisted with this project.

B. ADH STABILITY IN BLOOD:

In order to devise a satisfactory method of preserving ADH activity in blood samples, the mechanism of ADH destruction in whole blood was studied.

1. The Effect of Temperature on ADH Destruction

Several hundred ml. of blood were withdrawn from a dog and enough ADH was added to obtain a concentration of approximately 50 micropressor units (μU)/ml. Within one minute of the addition of ADH, 10 ml. samples of blood were placed in 12 ml. screw capped culture tubes and placed in the appropriate temperature environment for storage. At the same time five 10 ml. samples were immediately precipitated with 2 volumes of cold 12% trichloroacetic acid (TCA) and processed in the manner currently utilized for ADH assay in this laboratory. These samples were designated time zero and were considered to represent the initial concentration ($[C]_o$) of ADH in the pool.

The results of the initial study, in which samples were stored at 24°C, 37°C, and quick frozen in liquid nitrogen and stored at -60°C, are listed in Table I.

TABLE I
EFFECT OF TEMPERATURE ON ADH STABILITY
E 19

TIME	24°C			37°C			-60°C		
HR	[C]	$\%[C]_o$	K	[C]	$\%[C]_o$	K	[C]	$\%[C]_o$	K
0	46.0	100.0	-	46.0	100.0	-	46.0	100.0	-
24	10.8	23.5	.0603	4.86	10.6	.0935	8.25	17.9	-
45	6.70	14.6	.0423	0.86	1.8	.0876	11.4	24.8	-
189	-	-	-	-	-		13.6	29.6	-
309	-	-	-	-	-		25.3	55.0	-

T = 0. [C] EQUALS MEAN OF 47.3, 48.6, 41.0, AND 47.2
$K = (1/T)2.303 \log_{10}([C]_o/[C])$
[C] AND $[C]_o$ EXPRESSED AS UU/ML OF ADH IN BLOOD

Some difficulty with breakage of the glass tubes was encountered during the quick freezing process. This difficulty was solved by freezing in polypropylene tubes. It was evident that rate of destruction was approximately first order and that more observations should be made between t = 0 and t = 24 hrs. The low results obtained from the frozen samples were unexpected and will be discussed in Section 4.

The study was repeated with samples having been stored at 4°C, 24°C, and 37°C for periods varying between 0.5 to 16 hrs. The times of storage were selected exponentially in order to gain as much information as possible about early phases, even though the expectation of a first order decay would dictate a linear sampling interval. The results are listed in Table II.

EFFECT OF TEMPERATURE ON ADH STABILITY

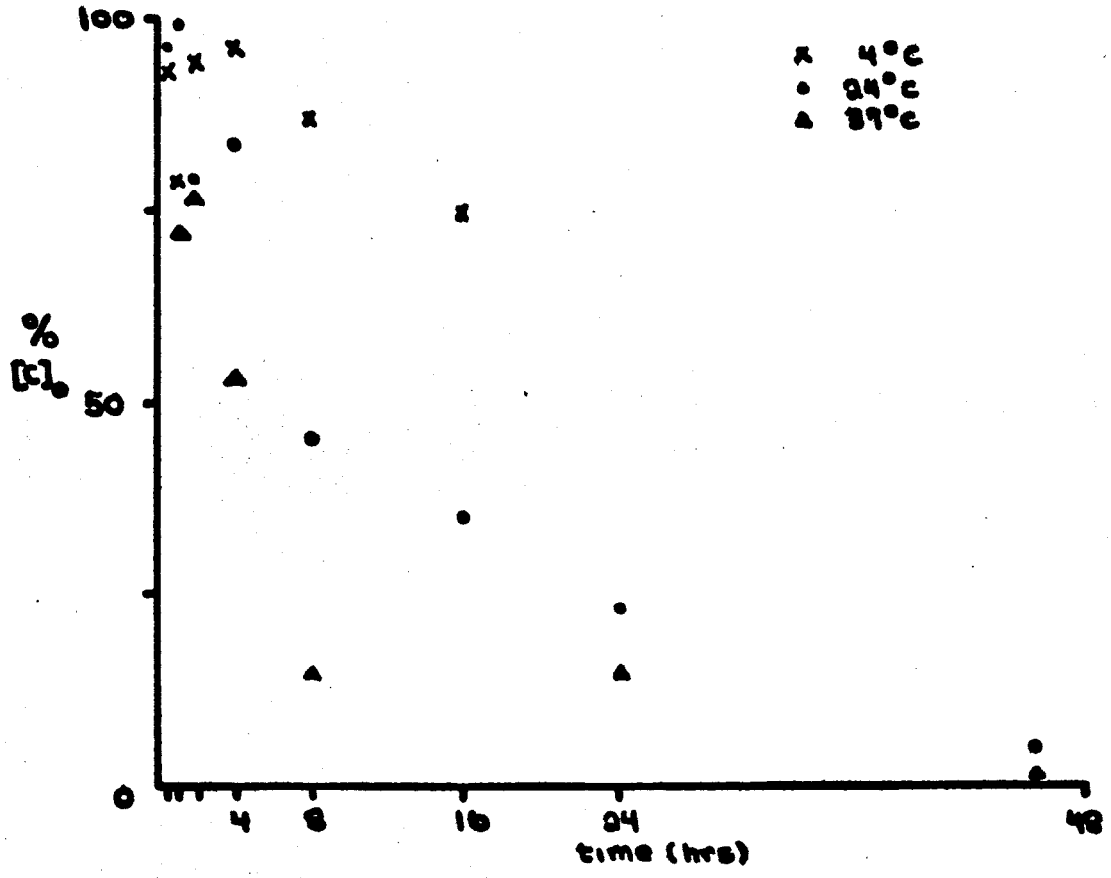

FIGURE 1

Figures 1 and 2 demonstrate the relationship between time and temperature on the rate of ADH decay.

TABLE II
EFFECT OF TEMPERATURE ON ADH STABILITY
E 23

TIME	4°C			24°C			37°C		
HR	[C]	%[C]$_o$	K	[C]	%[C]$_o$	K	[C]	%[C]$_o$	K
0	48.1	100.0	-	48.1	100.0	-	48.1	100.0	-
.5	44.9	93.4	.1372	46.3	96.2	.0305	-	-	-
1	40.1	83.4	.1818	48.0	99.8	.0020	34.9	72.5	.3214
2	45.3	94.1	.0305	40.2	83.6	.0895	39.9	82.9	.0937
4	46.0	95.7	.0076	40.3	83.8	.0441	25.7	53.4	.1569
8	42.1	87.5	.0167	22.0	45.8	.0976	14.7	30.6	.1480
16	35.2	73.2	.0195	16.7	34.7	.0661	-	-	-

T = 0. [C] EQUALS MEAN OF 46.1, 46.2, 49.9, 52.4, AND 45.9
K = (1/T)2.303LOG10([C]$_o$/[C])
[C] AND [C]$_o$ EXPRESSED AS UU/ML OF ADH IN BLOOD

EFFECT OF TEMPERATURE ON ADH STABILITY

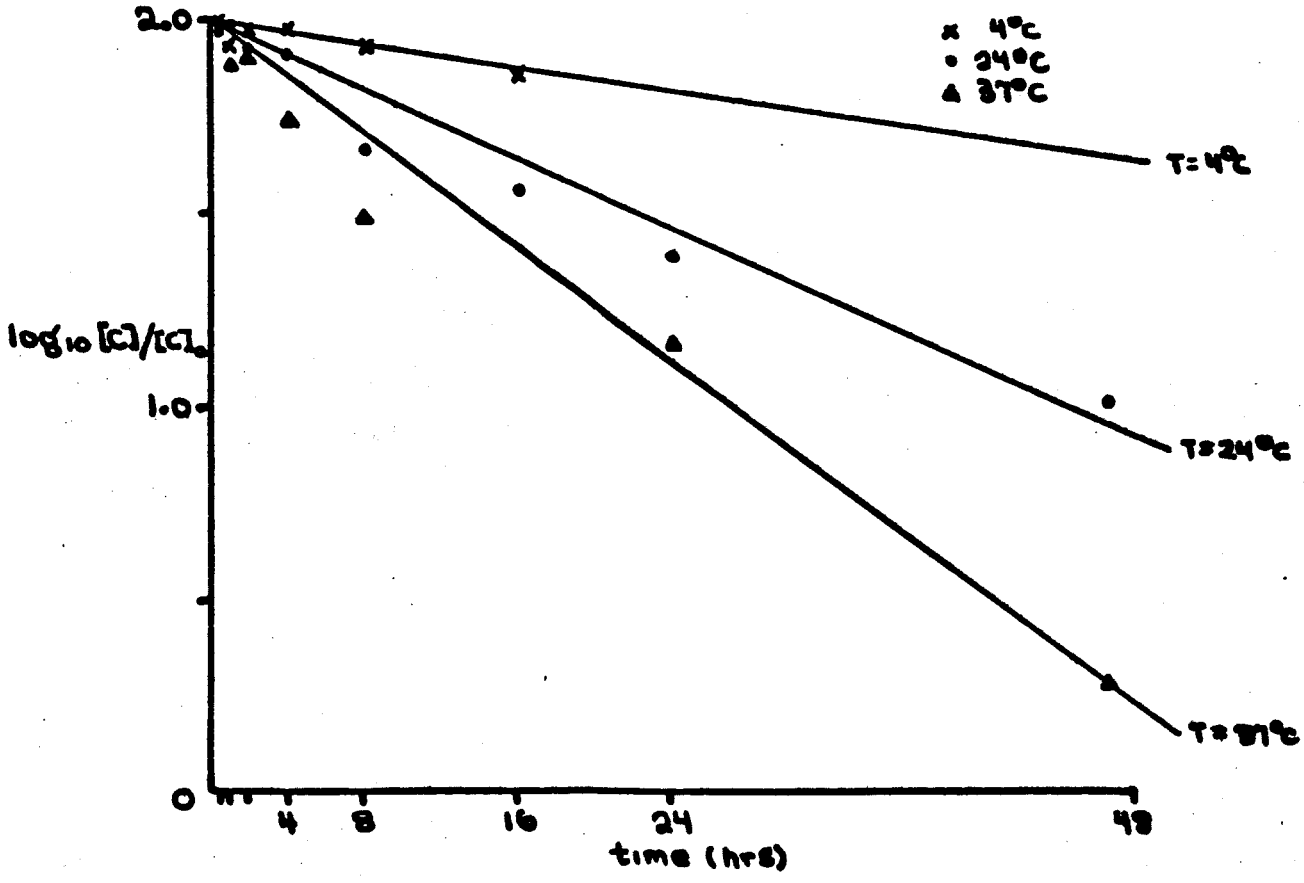

FIGURE 2

While the rate of decay may not be exactly first order, the present data are not inconsistent with a first order decay. I doubt that the extra work required to accurately determine the rate of reaction is justified at the present time. Calculations utilizing first order kinetics reasonably predict the values found. The effect of temperature is summarized in Table III and Figure 3.

TABLE III
EFFECT OF TEMPERATURE ON ADH STABILITY
E19 AND E23

T°C	K HR^{-1}	T HR	T°K	1/T	LOG10 K
4	.0182	38.0	277	.00361	-1.74
24	.0513	13.5	297	.00336	-1.29
37	.0839	8.3	310	.00322	-1.08

EFFECT OF TEMPERATURE ON ADH STABILITY

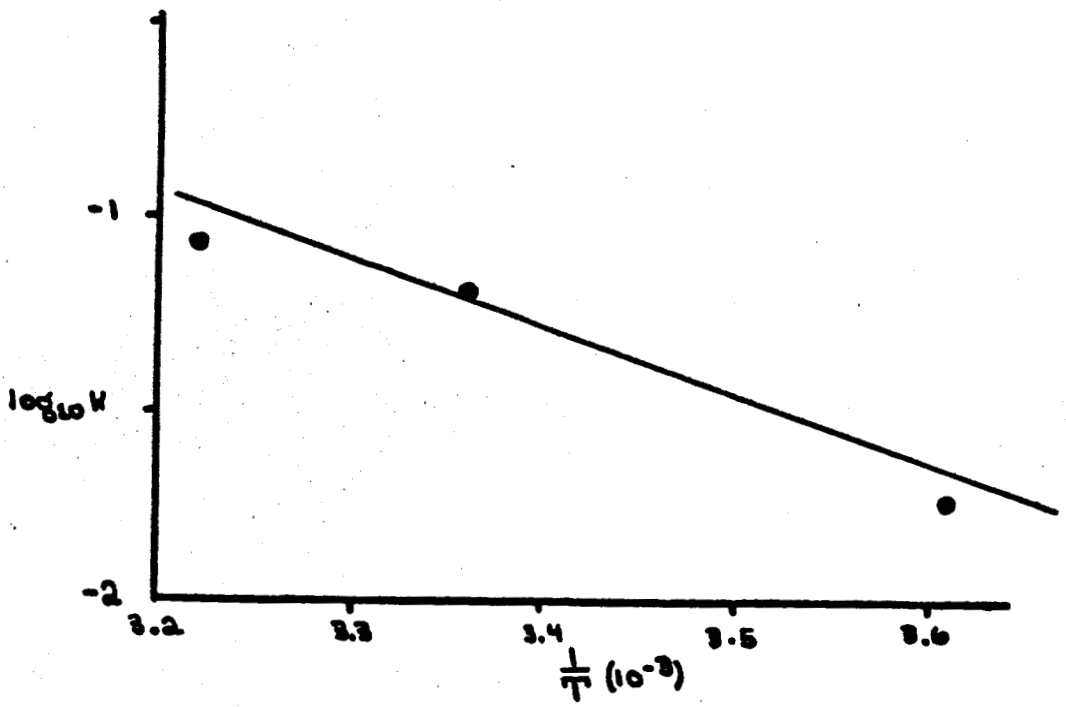

FIGURE 3

It appears that immediate cooling of the blood sample in ice would allow ample time for manipulations such as centrifugation for the separation of plasma without undue destruction of ADH. At the moment one must be cautious about freezing samples.

2. The Effect of pH on ADH Destruction

The lowering of the pH of a solution generally has a stabilizing effect on ADH. However, the study of the effect of pH was abandoned because it was impossible to lower the pH to an effective level and still maintain the physical characteristics of whole blood. This approach will be resumed in the studies of ADH stability in plasma and laked blood.

3. The Effect of Chelating Agents on ADH Destruction

Previous studied in this laboratory have demonstrated the protective effect of disodium ethylenediaminetetraacetate (2Na-EDTA) on ADH stability. In order to compare the effectiveness of 2Na-EDTA (12 mg./ml.), and a combination of EDTA and sodium oxalate (10 mg./ml.) in altering the $t_{\frac{1}{2}}$ of ADH at 24°C, storage studies were carried out in the manner described in Section 1. (See Table IV)

TABLE IV
EFFECT OF CHELATING AGENTS ON ADH STABILITY
E39 T = 24 C

	NO CHELATORS			EDTA 10^{-3}M			EDTA + OXALATE 10^{-2}M		
TIME HRS	[C]	K HR^{-1}	T HR	[C]	K HR^{-1}	T HR	[C]	K HR^{-1}	T HR
	51.9								
	54.0								
	55.5								
	61.5								
	55.1								
0	55.6								
	12.8			26.6			26.2		
	12.5			28.2			24.8		
	14.3			31.3			24.0		
	14.8			26.5			27.5		
24	13.6	.0587	11.8	28.2	.0283	24.5	25.5	.0325	21.3

The use of 10^{-3}M EDTA concentration significantly reduced the rate of ADH decay. Sodium oxalate did not interfer with the effectiveness of EDTA. This is a very important observation because the addition of sodium oxalate speeds the sedimentation of the red cells and improves the yield of plasma. Further studies are underway to determine the effectiveness of EDTA at 4°C and effectiveness of other inhibitors.

4. The Effect of Freezing on ADH Stability

The poor recovery experienced with samples stored in the frozen state prompted the further study of this particular problem. The increasing recovery of ADH (Table I) as greater care was taken during the thawing process in the 189 hr. and 309 hr. samples suggested that either a physical problem of dispersion or the release of destructive enzymes from the ruptured red cells might be involved. In order to test these possibilities a pool of fresh dog plasma was obtained. A 5 ml. aliquot was withdrawn and immediately processed. The pool was then slowly frozen at -20°C and stored at -20°C. At various intervals the pool was slowly thawed and a 5 ml. aliquot removed for ADH assay and the pool then refrozen. The data in Table V demonstrates the variability encountered and strongly supports the view of physical dispersion. The coefficient of variation of the plasma samples was the same as that found in the whole blood frozen pool samples and therefore rules out the release of enzymes from the red cells. In order to test the possibility that freezing disrupts the configuration of the ADH molecule, the solution which is used as a bioassay standard (16 µU of ADH/200 µl) was treated in the same manner as the plasma pool (see Table V).

TABLE V
EFFECT OF REPEATED FREEZING AND THAWING ON ADH STABILITY

	E21 PLASMA	E44 STD SOLUTION
FRESH	13.1	15.4
1ST	29.8	18.8
2ND	15.5	16.5
3RD	9.2	11.1
4TH		13.9
5TH		17.7
6TH		15.8
7TH		14.3
	16.8 CV = 53	15.4 CV = 15

The sample mean of 15.4 μU of ADH/200 μl. would indicate that repeated freezing and thawing had no effect on the ADH content of the standard solution. Some subtle change has occurred in this solution as indicated by the excessive coefficient of variation. Therefore the effect of freezing on blood ADH levels is probably due to physical factors that may be overcome by prevention of the change in physical state. This could be accomplished by the addition of "anti-freeze" and thereby take advantage of the protective effect of low temperatures. Until these techniques are worked out and tested, freezing of whole blood and plasma should be avoided. At the present time, if one wishes to accept the increased variability, the use of frozen plasma samples containing 10^{-3}M 2Na-EDTA is the best alternative to immediate processing.

C. CO-OPERATIVE STUDIES:

During the summer we have obtained blood samples containing high levels of ADH and have shipped them and their extracts to Dr. Evelyn Anderson, NASA, Ames Research Center, Moffett Field, California, for the estimation of corticotrophin releasing factor by her method. An experiment was designed to produce samples of high ADH content and to yield some information about the dynamics of the most potent stimulator of ADH release, hemorrhage. An anesthetized dog was slowly bled until his central arterial pressure was about 40 mm. of Hg. A total of 1400 ml. of blood was removed. All shed blood was saved in 100 ml. fractions and immediately processed as plasma. A 5 ml. aliquot of each 100 ml. sample was frozen. The rest of the sample was processed immediately and the extract frozen. ADH assays were carried out on a small aliquot of the extract. The effect of hemorrhage on plasma ADH levels is illustrated in Figure 4. The frozen plasma and their corresponding extracts were then sent to Dr. Anderson. She was unable to find any releasing factor in the extracts that contained ADH activity, although large amounts of releasing factor were found in the plasma from which the extracts were obtained. Since the isolation procedure that we employ preserves most of the ADH activity in the plasma a method has possibly been found for the removal of the interfering ADH.

EFFECT OF HEMORRHAGE ON PLASMA ADH LEVELS

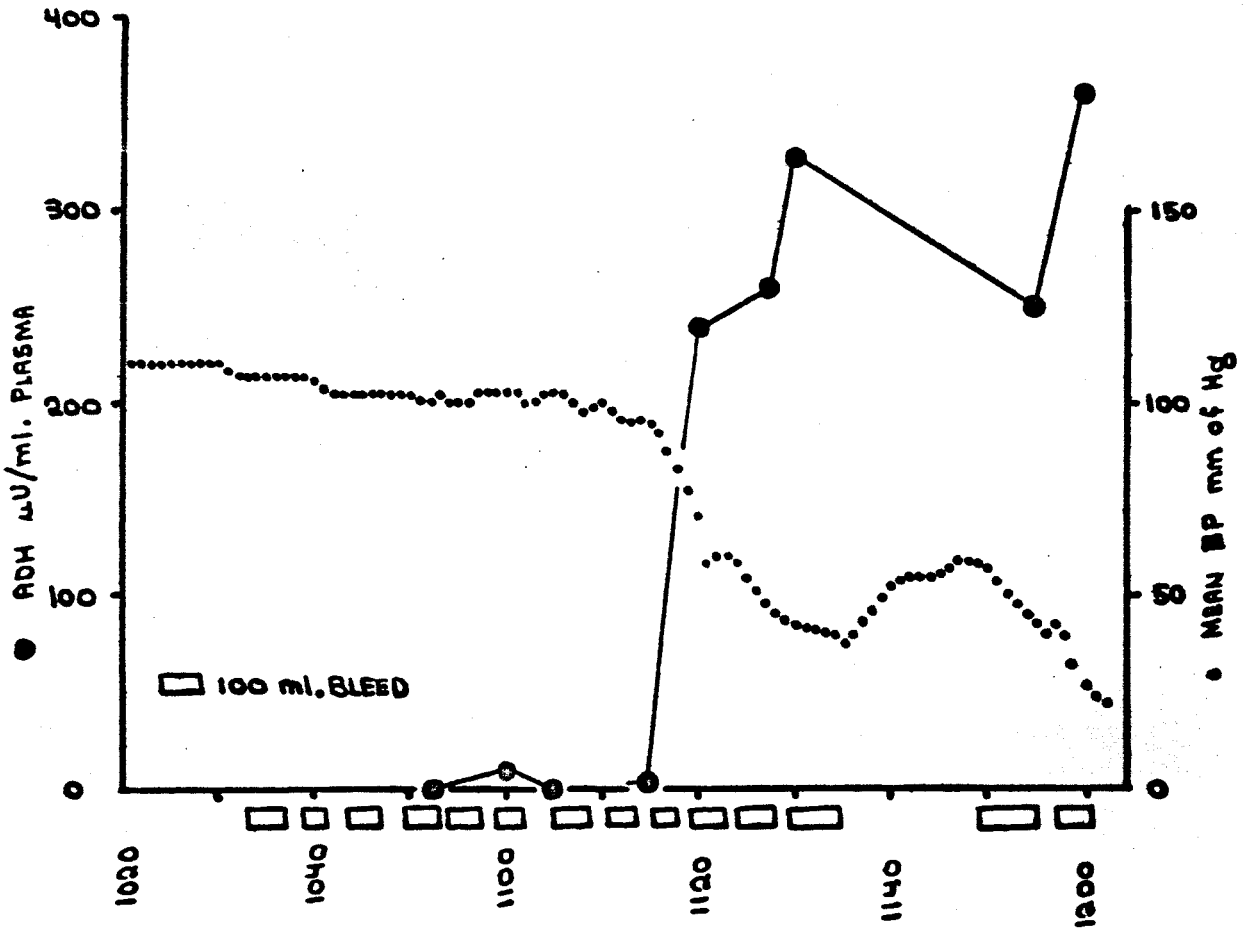

FIGURE 4

Recently, samples of human blood containing high concentrations of ADH in which the fractions coming from the isolation procedure have been saved, have been forwarded to the Ames Research Center in order that the behavior of the releasing factor on the chromatography columns can be ascertained.

D. SUMMARY:

Present work can best be summarized by emphasizing that the best results are obtained when blood samples for ADH assay are processed immediately (within one minute). The lowering of the sample temperature and the addition of EDTA may prove very useful in developing suitable alternatives to immediate processing.

See the Appendix for the suggested procedure to be used for obtaining blood samples to be sent to this laboratory for ADH assay. This procedure is judged to be the best currently available and will be changed as new techniques are developed.

No publications have resulted from this project as yet.

APPENDIX

Suggested Method of Processing Blood Samples Prior to Shipment to a Distant Laboratory for Antidiuretic Hormone Assay.

1. Withdraw 10 to 20 ml. of blood from a peripheral vein into a heparinized syringe and gently mix.

2. Quickly add this blood to a centrifuge tube containing enough powdered sodium oxalate to achieve a final concentration of 1 mg./ml. of blood and enough powdered disodium ethylenediaminetetraacetate to produce a final concentration of 1 mg./ml. of blood; and gently mix until all of the white powder is dissolved.

3. If there is to be a delay before the plasma is separated, cool the centrifuge tube that is filled with anti-coagulated blood by placing it in a beaker of crushed ice.

4. Centrifuge sample for 5 minutes and remove plasma.

5. Freeze plasma and ship in frozen state.